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The hypothesis to be tested in this application is whether STAT family of transcription factors play a role in the development of ER-negative breast carcinomas. During the past year, we have examined the activation status of two STATs, STAT-3 and STAT-5, known to be associated with the proliferation of normal and cancerous breast cell lines. Our results indicate that in a vast majority of theER-negative breast tumor cell lines, STAT-3 exists in a constitutively activated state and is localized in the nucleus. Normal human cell lines do not exhibit such an activated state or nuclear localization of STAT-3. Co-immunoprecipitation studies show that Src kinases immnunoprecipitate along with STAT-3 suggesting a physical association between these two proteins. These results suggest that several of the ER-negative breast carcinomas express constitutively activated Src kinases, which mediate the phosphorylation of STAT-3, which in turn could mediate their proliferative function and oncogenicity. The constitutively activated status of STAT-3, therefore serves as a good diagnostic marker for the detection of ER-negative breast carcinoma development. Since STAT-3 activation appears to play a critical role in the proliferative function of ER-negative breast carcinomas, Src-STAT-3 pathway offers an excellent target for the development of cancer therapeutic agents.

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Table of Contents

Introduction

STATs (Signal Transducers and Activators of Transcription) are a group of highly related transcription factors, which were originally described by Darnell and his co-workers as ligand-induced transcription factors in interferon-treated cells. Subsequent studies by a number of groups showed that STATs play a critical role in signal transduction pathways associated with several growth factors, cytokines and neurokines. Todate, seven mammalian genes that code for different STATs have been identified, all of which encode for proteins of 750-850 amino acids long and are characterized by the presence of a DNA-binding domain followed by putative SH3 and SH2 domains. These proteins, which are normally localized in the cytoplasm, are activated when phosphorylated on a single tyrosine located around residue 700, which facilitates their dimerization and translocation to the nucleus. The phosphorylation of STATs is known to occur immediately after the binding of growth factors or interferons to their receptors. Since this receptor-ligand interaction was also found to result in the activation of JAK kinases, which often exist in association with cytokine receptors, it was originally thought that STATs might be substrates for JAK kinases. However, our recent studies show that interaction of IL-3 with its receptor leads to the activation of c-Src kinase activity, which in turn facilitates the binding of c-Src to STAT-3. This association leads to the phosphorylation of STAT-3, allowing this transcription factor to translocate to the nucleus. Expression of a dominant negative mutant of *src* (AMSrc) in these cells results in a block to IL-3 mediated phosphorylation of STAT-3, and its ability to bind to DNA. On the other hand, expression of a dominant negative mutant of JAK2 (JAK2KE) had no effect on IL-3-mediated activation of STAT-3. Our results also show that AMSrc does not affect the phosphorylation of JAK2, suggesting that two independent pathways mediate JAK and STAT phosphorylation events. Inhibition of c-Src activation by AMSrc, which leads to a block to STAT-3 activation, results in a dramatic inhibition of

cell proliferation mediated by IL-3. These results suggest that Src family kinases mediate the phosphorylation of STATs and play a critical role in signal transduction pathways associated with myeloid cell proliferation.

It is now well established that the estrogen receptor status of primary breast tumors is of considerable value to the clinician in designing protocols for treatment. The retention of the estrogen receptor in breast tumor cells suggests a more differentiated state of the neoplasia and often suggests better prognosis and longer survival. An important question that remains to be addressed is the identification of the signal transduction pathway that is associated with the proliferative and metastatic potential of the ER^- breast tumors. A comparative analysis of signal transduction pathways in ER+ and ER- breast tumors in our lab led to the observation that all of the ER- breast tumors express an activated (Tyrosine phosphorylated) form of STAT-3. Interestingly, ER+ breast tumors do not express such an activated form suggesting that these tumors are not dependent on this pathway for their proliferative potential.

In our application, we proposed to examine the molecular basis for the activation of STAT-3 and design approaches to block the activation of this transcription factor and examine the effects on deactivation of this pathway on ER- breast tumor cell growth. We proposed to specifically address the following questions. (1) What is the mechanism of activation of STAT-3 in ER- breast carcinomas? (2) How is c-Src activated in ER- breast carcinomas? (3) Is there a correlation between Erb-B2 expression and STAT-3 activation? (4) Does a dominant negative mutant of STAT-3 block ER- breast tumor cell proliferation? and (5) What is the nature of genes that are activated as a result of STAT-3 activation in ER- breast carcinomas?

Body

STAT-3 is constitutively activated in human breast carcinomas, which are estrogen receptor negative

Recent studies suggest that STATs play an important role in the proliferation of breast epithelial compartment, suggesting that this pathway may play a critical role in tumorigenesis. To examine the role of JAKs and STATs in the proliferation and oncogenic behavior of human breast tumor cells, we examined the activated status of STAT-3 and STAT-5 in several of the human breast carcinomas. In addition to the tumor cell lines, we used HS578BST cell line, which is derived form normal breast tissue as a control. For this, we performed a western blot analysis of the total proteins extracted from these breast carcinomas, which have been subjected to electrophoretic separation on acrylamide gels followed by a transfer to nytran membranes. This included a panel of ER+ and ER- tumor lines and to assess their ER status, the western blots were probed with an antibody to ER. Results from this experiment showed that MCF-7, BT474, T47D, 415, MDA-MB-330, and ZR75-30 express ER while BT20, 435, MDA-MB-435S, MDA-MB-468, MCF10F, and HTB126 were found to be ER-negative. Probing of these western blots with an antibody that recognizes the STAT-3 protein showed that all of them express approximately equal amounts of STAT-3. However, when the same blot was stripped and re-probed with an antibody that specifically recognized the Tyrosine-phosphorylated form of STAT-3 (which is activated), only the ER-negative tumor cell lines exhibited a constitutively phosphorylated form of STAT-3. These results suggest that the proliferative potential of the ER^- breast carcinomas could be mediated by the activated STAT-3 pathway.

A similar analysis of these tumor cell lines for the presence of STAT-5 revealed that this

transcription factor, like STAT-3, is abundantly expressed in all of the breast carcinomas tested. However, an analysis of its activated status showed that, unlike STAT-3, this transcription factor also exists in a constitutively phosphorylated state in both ER+ and ER- cell lines. A consistent difference seen with these two classes of tumors was a higher level of STAT-5 phosphorylation in ER+ cell lines, which was approximately 2-3 fold higher than that seen with ER-ve cell lines. In contrast to the breast tumor cell lines, HS578BST cell line did not show the presence of either STAT-3 or STAT-5 in a phosphorylated state.

Are JAK kinases exist in a constitutively activated state in human breast carcinomas?

To determine whether the observed phosphorylation of STAT-3 and STAT-5 in human breast carcinomas correlates with the phosphorylation status of any of the JAK kinases, we immunoprecipitated cell extracts from both ER+ and ER- cell lines with JAK-specific antibodies. These immunoprecipitates were subjected to SDS-PAGE and the western blots were probed with respective JAK antibodies and monoclonal antibody 4G10, which specifically recognizes the phosphorylated tyrosine moiety. As a positive control, we used total cell extract prepared from 32Dcl3 cells that have been stimulated with IL-3. Results from this experiment show that all of the cell lines express readily detectable levels of both JAK-1 and JAK-2. However, neither of these kinases were found to exist in a constitutively phosphorylated state in any of the breast carcinomas tested. However, we could readily detect the phosphorylated form of JAK-1 and JAK-2 in our positive control, cell extracts derived from 32Dcl3 cell line that has been treated with IL-3. None of the breast tumor cell lines expressed detectable levels of Tyk-2 and JAK-3 (data not shown). These results demonstrate that the phosphorylation status of STAT-3 and STAT-5 does not correlate with the phosphorylation status of JAKs that are

expressed in these cell lines.

Expression of Src kinases in human breast carcinoma cell lines

Recent studies with NIH/3T3 cells as well as 32Dcl3 cells that are transformed by v-src have demonstrated that these transformed cells express constitutively activated form of STAT-3. In addition, the v-Src-mediated transformation of these cells can be reversed by the expression of a dominant negative mutant of STAT-3. These studies also provided evidence that the constitutive activation of STAT-3 is preceded by the physical association of v-Src with STAT-3. A similar association of c-Src protein was seen by us in normal 32Dcl3 cells that are activated by IL-3. To determine whether the observed activation of STAT-3 in ER- breast carcinomas is mediated by Src kinases, we first examined the expression pattern of c-Src and c-Fyn in human breast carcinomas. We chose these two kinases because these two tyrosine kinases that have been previously shown by us and others to bind to and phosphorylate STAT-3. Our results from these studies demonstrate that while c-src is expressed ubiquitously in all of the breast tumor cell lines. c-fyn is preferentially expressed in ER- cell lines.

We next examined whether c-Src and c-Fyn seen in human breast carcinoma cell lines associate with STAT-3 and whether this association is dependent on the ER status of the cell lines. For this, cell lysates were prepared form different human breast carcinoma cell lines and examined for the presence of c-Src, c-Fyn and STAT-3 by western blot analysis. We next immunoprecipitated these cell lysates with monoclonal antibodies against c-Src and c-Fyn and resolved the immunoprecipitates on 10% SDS-polyacrylamide gels. The gels were then blotted onto a nytran membrane and subjected to western blot analysis using antibodies against STAT-3. Results of these experiments show that c-Src immunprecipitates derived from ER- and ER+ breast carcinomas contain

associated STAT-3 protein, even though the ER+ cells do not contain phosphorylated form of STAT-3. Similar experiments with c-Fyn show that all ER- breast carcinomas, which express this protein exhibit co-immunoprecipitation of STAT-3. These results suggest that association of Src or Fyn with STAT-3 alone is not adequate for the phosphorylation of STAT-3 and a second molecular event that has gone awry in ER- cells contributes to the phosphorylated state of STAT-3 in these cells.

A dominant negative mutant of Src blocks activation of STAT-3 in ER- breast carcinomas

To definitively demonstrate that c-Src or c-Fyn mediate phosphorylation of STAT-3, we stably transfected BT-20 and 126 cell lines cells with a tetracycline-inducible dominant negative mutant of Src (AMSrc). The ATP-binding site of this c-*src* mutant was inactivated by mutation of lysine 295 to arginine rendering this protein kinase-inactive. In addition a phenylalanine substitution for tyrosine 527 prevents the intramolecular interaction between phosphorylated Y527 and the SH2 domain of this protein allowing the protein to exist in an open configuration, thus making the SH2 and SH3 domains accessible to cellular binding proteins. This protein was tagged with a peptide sequence derived from the hemaglutinin gene (the FLAG epitope) at the C-terminal end, which allowed the detection of AMSrc independent of endogenous c-Src. Following electroporation of the expression vector into BT-20 and HTB126 cell lines, the cells were selected with G418 and maintained in a medium containing 2μg/ml of tetracycline. To verify the inducible expression of AMSrc in transfected cells, they were incubated in a medium lacking tetracycline for 48 hrs and the cell lysates subjected to western blotting using anti-FLAG antibodies. These western blotting studies showed that the expression of AMSrc protein was very leaky in these cell lines and the presence or absence of tetracycline in the medium did not affect the levels of AM-Src expressed in

these cells. To determine the phosphorylation status of STAT-3 in cells expressing AMSrc, total cell lysates from normal BT-20 and HTB126 cells and AMSrc-transfected BT-20 and HTB126 cells were prepared. The lysates were immunoprecipitated with anti-STAT-3 antibodies and the immunoprecipitates subjected to western blotting. The western blots were first probed with anti-STAT-3 antibodies, which showed that all cell lines expressed equivalent amounts of STAT-3. The western blot was stripped and re-probed with anti-phospho-STAT-3 antibodies which specifically recognizes the phosphorylated form of STAT-3. These studies revealed that this antibody readily recognizes phosphorylated STAT-3 present in untransfected BT-20 and HTB126 cells while it failed to recognize STAT-3 in cell lysates derived from AmSrc-transfected BT-20 and HTB126 cells. These results show that phosphorylation of STAT-3 in ER- breast carcinomas is blocked by AMSrc indicating that constitutive phosphorylation of STAT-3 in ER- breast carcinomas is mediated by a Src kinase.

Key Research Accomplishments:

- STAT-3 protein exists in a constitutively activated state in ER-negative breast carcinomas.
- STAT-3 phosphorylation in ER-negative breast carcinomas is mediated by Src-family of tyrosine kinases.
- ER+ breast carcinomas do not express constitutively activated form of STAT-3.
- ER+ breast carcinomas express higher levels of activated STAT-5 compared to ER-negative breast carcinomas.
- A dominant negative mutant of c-Src blocks the phosphorylation of STAT-3 in ER-negative breast carcinomas.

Reportable Outcomes:

The data obtained from the DOD grant was used in an application for NIH RO1 funding and a grant entitled "Role of Myb gene family in breast cancer" was approved for funding by NIH.

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Conclusions:

Our results suggest that several of the human breast carcinomas, which are ER-negative express constitutively activated form of STAT-3. STAT-3, in these tumors appears to be associated with an activated form of Src kinases, which in turn mediate the phosphorylation of STAT-3. The constitutively activated status of STAT-3, therefore serves as a good diagnostic marker for the detection of breast carcinoma development. Since STAT-3 activation appears to play a critical role in the proliferative function of human breast carcinomas which are ER-negative, Src-STAT-3 pathway offers an excellent target for the development of new cancer therapeutic agents.